THE EFFECT OF pH UPON THE BACTERIOSTATIC ACTIVITY OF CERTAIN NITROPHENOLS[1]

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Although it is generally realized that the bacteriostatic activities of acidic substances as a rule increase as the pH of the culture medium is lowered, there appears to be in the literature no information on the rates at which such changes occur in terms of the concentrations and the ionization constants of the substances and the pH of the medium. In this paper are presented some data on the bacteriostatic powers of a number of nitrophenols for which the inhibiting concentrations at different culture pH values were determined.

As test organisms cultures of *Escherichia coli* and *Bacillus mesentericus* were used, and for media both extract broth and simple chemically defined media were employed. Since the results with the two species and the two media were essentially the same, only the data obtained with *E. coli* grown in extract broth will be given.

The broth and the broth solutions of the compounds were adjusted with HCl or NaOH to pH values of 5.5, 6.5, 7.5, and 8.5. The concentrations of the substances under test were, as a rule, decreased in 3:4 ratio by transferring 15-ml volumes through tubes containing 5 ml of broth. This gives a logarithmic reduction in concentration to $\frac{1}{10}$ of the original value through a series of nine tubes. The inoculum consisted of one drop of a 1:1,000 dilution of an 18-hour broth culture, and incubation was at 37 C.

The bacteriostatic value of a substance was taken as the lowest molar concentration preventing visible growth for 4 days, and the final value used was the geometric mean of several determinations, usually four or five. These figures are included in the following table of the compounds with their pKa constants.

An examination of the graph, from which the curves of several of the compounds have been omitted for the sake of simplicity, shows that, in general, the more acid a substance is, the greater is the effect of pH change upon its bacteriostatic power. *m*-Nitrophenol, with a pKa of 8.3, is but little affected throughout the range of culture pH studied, whereas a compound such as 2,5-dinitrophenol, pKa 5.1, increases rapidly in potency as the pH falls. The behavior of picric acid may be due in part to the fact that it is present almost exclusively in the ionic form, and, as a rule, cell membranes are relatively impermeable to such anions. The irregular action of picric acid is duplicated at a high pH (8.5) by the other more acidic compounds.

The behavior of the nitrophenols contrasts markedly with that of the large series of acridine derivatives studied by Albert and his colleagues (1945). The

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latter substances seem to owe their activity to the cation, so that the most basic and hence most highly dissociated compounds are the most effective bacteriostatic agents. The activity is directly proportional to the concentration of cations existing at the culture pH values studied and is apparently due to com-

TABLE 1

	pKa	MOLAR CONCENTRATIONS INHIBITIVE AT pH VALUES OF			
		5.5	6.5	7.5	8.5
m-Nitrophenol	8.3	.0028	.0026	.0028	.005
p-Nitrophenol	7.1	.0007	.001	.0025	.0044
2-Amino,4-nitrophenol	7.0	.002	.0033	.008	.025
2,5-Dinitrophenol	5.1	.00011	.00035	.0011	.0044
2-Amino,4,6-dinitrophenol	4.4	.00056	.003	.011	.02
2,4-Dinitrophenol	4.0	.00028	.0011	.0056	.016
2,6-Dinitrophenol	3.6	.00032	.0016	.011	.018
2,4,6-Trinitrophenol	0.8	.0035	.009	.014	.014

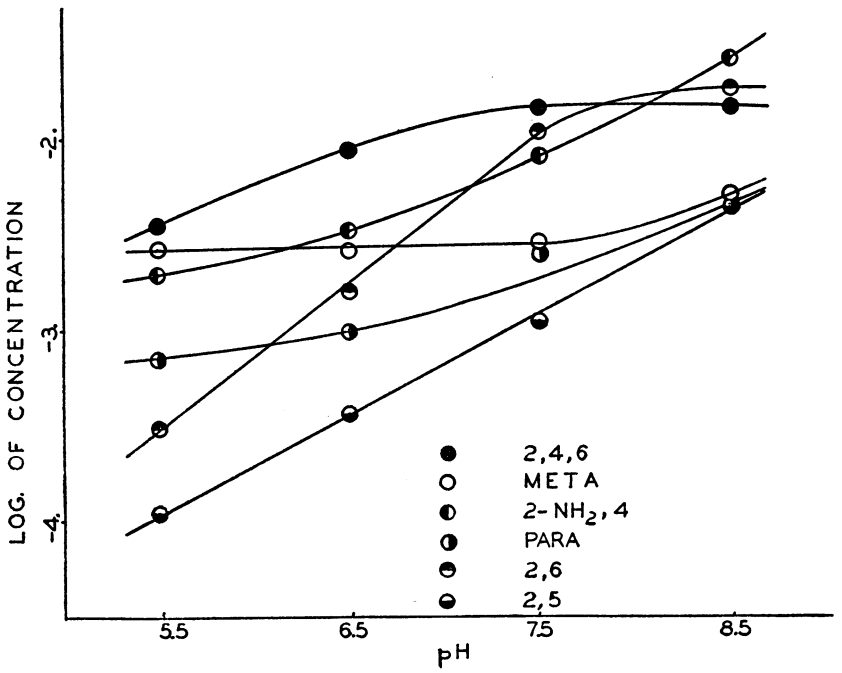

Figure 1. Bacteriostatic action of nitrophenols.

petition of these with hydrogen ions for some position on an enzyme surface. Since the oil-water partition coefficients of the active acridines are close to unity, it is deduced that the bacteriostatic action takes place in the aqueous phase, probably at the cell surface.

With the nitrophenols, on the other hand, there is no apparent relationship

between the concentrations of the anions of the bacteriostatic substances and the concentration of hydroxyl ions. This, together with the decrease in efficacy as the anion: molecule ratio increases, suggests that the substances owe their activity to the undissociated compound.

In an effort to explain this behavior of the nitrophenols by established chemical principles, the data have been examined from the standpoint of the law of mass action. An approach similar to that used previously (Klotz, 1944) in an analysis of sulfonamide action has yielded expressions that are in semiquantitative agreement with the observations recorded in this paper.

For the simplest treatment it has been assumed that the phenol is active in its nonionized form, that no significant fraction of it is removed by the bacterial enzymes or other proteins, and that the effect of pH on these proteins may be neglected. The first assumption is supported by the results reported in this paper and by the work of Krahl and Clowes (1938), who found, with similar substituted phenols, that the inhibition of cell division in the case of *Arbacia punctulata* eggs is dependent on the concentration of the phenol molecules in the external medium, and hence in the cell, and is independent of the concentration of the anions. The second assumption is, of course, quite arbitrary and is made only as a first approximation to simplify the theoretical treatment. This assumption is eliminated in the second treatment, which is described below. The third assumption is based on a picture of binding occurring by a hydrogen bridge between the nonionized phenol molecule and an anionic carboxylate group on the protein. In the region under investigation, pH 5.5 to 8.5, the carboxyl groups are completely ionized and not subject to modification by changes in acidity.

For the purposes of the following derivation we may set up these definitions:

HD = nonionized phenol
D^- = ionized phenol
$C = HD + D^-$ = total phenol required to produce bacteriostatis.

From the law of mass action one may write the equilibrium expression

$$\frac{(H^+)(D^-)}{(HD)} = K \tag{1}$$

where K is the ionization constant of the drug. It follows immediately with the aid of equation (1) that

$$C = \frac{(H^+)(D^-)}{K} + (D^-) \tag{2}$$

It is desired to know the variation of C with (H^+) under conditions such that (HD) is kept constant and bacteriostasis is maintained. For this purpose one may differentiate equation (2) with respect to (H^+) to obtain:

$$\frac{dC}{d(H^+)} = \frac{H^+}{K}\frac{d(D^-)}{d(H^+)} + \frac{(D^-)}{K} + \frac{d(D^-)}{d(H^+)} \tag{3}$$

Since

$$(D^-) = C - (HD) \tag{4}$$

then

$$\frac{d(D^-)}{d(H^+)} = \frac{dC}{d(H^+)} \tag{5}$$

since (HD) is constant. Hence equation (3) becomes

$$0 = (H^+) \frac{dC}{d(H^+)} + (D^-) \tag{6}$$

Consequently, one may obtain the expression

$$\frac{(H^+)}{C} \frac{dC}{d(H^+)} = \frac{d\ln C}{d\ln(H^+)} = -\frac{(D^-)}{C} = -\frac{(D^-)}{(HD) + (D^-)} = \frac{K}{K + (H^+)} \tag{7}$$

Therefore,

$$\frac{d \log C}{d\ ph} = \frac{K}{K + (H^+)} \tag{8}$$

From equation (8) it is evident that when the drug is a very weak acid, (H^+) in the culture range is always greater than K and the slope of the log C–pH line will be a small number approaching zero. This is the observed behavior with *m*-nitrophenol (pK 8.3) until pH 8.5, at which (H^+) and K are comparable in size and the slope should, and does, increase. For such compounds as *p*-nitrophenol (pK 7.1) and 2-amino-4-nitrophenol (pK 7.0) where the pK values fall in the culture pH range, the slope should tend to rise as the culture pH rises. This is seen to be the case. With the very acid compounds, (H^+) in the denominator of equation (8) is negligible and hence the slope should be 1 and independent of pH in the culture range. For 2,5-dinitrophenol (pK 5.1) and 2,6-dinitrophenol (pK 3.6) the slopes are substantially constant except perhaps at high pH for the latter drug. On the other hand, the experimental observations definitely depart from theory in that the slopes for these very acid phenols are not unity, though they do tend to increase toward 1 as the acidity of the drug increases. The largest slope found in this study is 0.75, with 2,6-dinitrophenol.

The basis of this discrepancy probably lies in the assumption that none of the phenol is removed by the bacteria. It has seemed appropriate, therefore, to develop an expression for the slope of the log C–pH plot which takes into account the possibility that a significant portion of the nonionized form may be bound by the bacteria. For this purpose we must revise our definition of C to

$$C = HD + D^- + PHD$$

and we define T, the total protein present, by the expression

$$T = P + PHD$$

Since we are now considering two equilibria, we must set up two equilibrium constants

$$\frac{(H^+)(D^-)}{(HD)} = K \tag{1}$$

$$\frac{(HD)(P)}{(PHD)} = k \tag{9}$$

With these definitions and equilibrium expressions, one can derive the following equation for the slope, by a procedure analogous to that described above, if we assume that bacteriostasis is maintained by keeping constant values of bound phenol, PHD.

$$\left(\frac{d \log C}{d\ pH}\right)_{\text{PHD constant}} = \frac{K}{K + (H^+)\left[1 + \frac{P}{k}\right]} \tag{10}$$

Since this expression contains two parameters, P and k, which are unknown, it is not possible to predict explicitly the value of the second term in the denominator. Nevertheless, it is obvious that the additional factor (1 + P/k), would tend to increase the magnitude of the denominator and hence make the slope less than 1 even for very acid compounds. It is thus evident that this more detailed analysis gives better agreement with the experimental observations.

Consideration has been given also to the possibility that the phenol may not be bound to carboxylate ions but rather to a basic nitrogen atom on the protein. In such a situation the assumption of pH independence on the part of the protein might be erroneous. If one wishes to take pH effects into account, it is necessary to introduce a third equilibrium constant, k_2, to correlate the acid properties of the protein. It is convenient to define k_2, and the auxiliary constants K′ and k′ in the following manner:

$$\frac{(HP^+)}{(H^+)(P)} = k_2 \tag{11}$$

$$\frac{(HD)}{(H^+)(D^-)} = K' = \frac{1}{K} \tag{12}$$

$$\frac{(PHD)}{(P)(HD)} = k' = \frac{1}{k} \tag{13}$$

By differentiation and algebraic manipulation similar to that described already one obtains a more detailed expression for the slope of the log C − pH curve

$$\frac{d \log C}{d\ pH} = \frac{1 - K'k_2(H^+)^2}{[1 + k_2(H^+)][1 + K'(H^+)] + K'k'(H^+)[T - (PHD)]} \tag{14}$$

It is apparent, however, that this relationship is quite unwieldy and hence of little immediate value in connection with the present problem.

CONCLUSIONS

For most members of a group of nitrophenols examined, the changes in bacteriostatic potency at different culture pH levels can be correlated with the acid strengths of the compounds. A weakly acidic substance (pKa between 8 and 9) is inhibitive in almost the same concentration throughout a culture pH range of 5.5 to 8.5, whereas a more acid compound (pKa in the neighborhood of 4, for example) may undergo a 60-fold change in potency through the same range. The acitivity seems to be due to the undissociated molecule of the drug.

By a mass law approach a simple formula may be derived that gives semi-quantitative agreement with the experimental findings. Two other formulae are offered, which would in all probability agree much more closely with these facts, were the proper equilibrium constants available.

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